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Award Number: W81XWH-06-2-0019

TITLE: Molecular Connections between Arousal and Metabolic Disease: Orexin and Modafinil

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REPORT DATE: April 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2008	Annual	27 MAR 2007 - 26 MAR 2008

4. TITLE AND SUBTITLE

Molecular Connections between Arousal and Metabolic Disease: Orexin and Modafinil

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-2-0019

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Cincinnati
Cincinnati, Ohio, 45237

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Obesity and metabolic diseases are known to be tightly linked to arousal-sleep cycles. Further, both metabolic disease and arousal are known to have significant impacts on cognitive function in humans and animals. Importantly, the armed forces represent a population at significant risk for increased stress and disrupted arousal-sleep cycles. Because the incidence of metabolic disease and obesity is increasing, even in these physically fit individuals, understanding the interactions between these systems is highly significant. Further, some anti-fatigue pharmacologies (e.g.,modafinil) are already used in military settings, though their long-term effects on metabolism or central nervous system function are not well-understood. We have completed Year 2 of the proposed funding period to assess the physiological and behavioral effects of this pharmacology on rat subjects and identify potential molecular mechanisms activated by nutrients. Our data demonstrate that chronic administration of intraperitoneal modafinil does not alter dietary induced obesity or impair glucose tolerance. Additionally, we observed that chronic central modafinil does not increase stress in rats, but does attenuate object recognition memory. Ongoing studies are assessing the effects of pharmacological inhibition or activation of a potential molecular mechanism, mTOR.

15. SUBJECT TERMS
Obesity, diabetes, insulin, orexin-A, arousal, stress, cognition.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	17	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction:

The incidence of obesity is escalating to epidemic proportions in all segments of society. Even among the military, with much higher levels of fitness than civilian populations, are experiencing a rapid increase in obesity and metabolic disease. Recent research has suggested an important connection between arousal/stress physiology and metabolism. One important basis for this connection may be the brain orexin-A system, which is also the principal target of the anti-fatigue drug modafinil. However, the specific molecular machinery remains unidentified, as do the behavioral effects of manipulating this system. **Objective/Hypotheses:** We have hypothesized that modafinil and other anti-fatigue drugs may act by modulating metabolic pathways in the central nervous system. We also hypothesize that chronic stress and disruption of arousal-sleep system leads to impaired metabolic function and increased susceptibility to obesity. Finally, we hypothesized that central metabolic pathways can be activated by foods and nutritional interventions, in lieu of pharmacological manipulation, with less risk of long-term metabolic complications. To assess these hypotheses, we are conducting several studies in rat subjects. The primary study deigns include administration of modafinil and assessment of the molecular and genetic effects (Project 1) as well as behavioral consequences (Project 2). During the second year of funding we have also pursued a suspected underlying metabolic pathway that might mediate the effects of nutrients on energy regulation as well as behavior. The mammalian target of Rapamycin (mTOR) kinase is a key regulator of several cellular functions, including cell growth and differentiation.

Body:

During the second year of funding, we have made significant progress toward the stated aims and objectives. All proposed studies for Year 2 have been completed and we detail the results and conclusions here. For each study, we list the statement of work task, specific objectives, methods employed, and results obtained. Figures referenced are presented in an appendix at the end of the report.

Project 1 Year 2 Tasks:

Task 1: Assess the metabolic effects of chronic orexin-A activation by modafinil (1-18 months)

- Assess chronic effects on food intake, energy expenditure, metabolic rate, and body adiposity.
- Measure insulin sensitivity and glucose homeostasis.
- Assess the effect of modafinil to exacerbate the metabolic effects of stress and circadian disruption.

Task 2: Identify metabolic pathways involved in modafinil action (19-36 months)

- Identify key nutrient sensitive molecules in orexin producing neurons.
- Determine whether nutrient sensitive pathways alter orexin neuronal activity.

Experiment Series 1.2 Methods

Adult, male, Long-Evans rats were used in all of these experiments. The choice of diets for these experiments was critical. In this case, we uses a diet that is 40% fat (by calories) with the predominant source of fat coming from saturated fat (1). We have a great deal of experience with this particular diet and it is much closer to a typical human diet than is standard rodent chow which is inconsistent in its ingredients and only 8% fat. Rats were given modafinil at a dose of 4.0 mg/kg once

per day by oral gavage. These doses are approximately equivalent to doses used to increase performance in sleep-deprived humans. The key end points to be measured were measures of glucose tolerance.

Experiment Series 1.2 Results

We observed that chronic modafinil administration significantly reduced body weight and body adiposity in rats. However, what this experiment does not tell us is whether modafinil might lead to other disruptions in metabolic functioning that might leave the warfighter using modafinil at greater risk for metabolic disease. This is particularly true because a large data base links increased orexin activity to metabolic dysfunction. Interestingly, when rats were given an IP glucose tolerance test, rats given modafinil showed a strong trend towards higher glucose levels (see Figure 1). This is particularly interesting because rats given modafinil actually had lower body weights and body fats. Thus, we would have expected glucose tolerance to actually improve in these animals making even the small observed degradation potentially quite important.

The next experiment was to test whether exposure to modafinil might result in increased susceptibility to weight gain when rats are subsequently exposed to a high-fat diet. This experiment would be analogous to a warfighter using the drug during training or combat and then potentially experiencing negative side-effects after returning to civilian life where exposure to high-fat foods are plentiful. While weight gain clearly accelerates when rats are exposed to the high-fat diet, there is no difference in weight gain between the rats that had been exposed to a high-fat diet from those that had not (see Figures 2 and 3).

Experiment Series 2.1 Methods and Results

Because hypothalamic mTORC1 signaling has been implicated as a target of leptin in the regulation of energy balance, we investigated its role in obesity-induced leptin resistance. In contrast to rats maintained on a low-fat (LF) diet for 3 weeks, rats maintained on a HF-diet had no anorexic response to icv leptin (Figure 4). Western blot analysis revealed that leptin was unable to modulate hypothalamic mTORC1 signaling in the HF group, whereas it significantly induced phosphorylation of both S6 Kinase 1 (S6K1) and S6 ribosomal protein (S6) in the LF group. Similar to leptin, the cytokine ciliary neurotrophic factor (CNTF) induces hypophagia and increases STAT3 phosphorylation. However, CNTF and its analogue CNTFAx15 activate leptin-like pathways in the hypothalamus even in leptin-resistant states, including diet-induced obesity. Icv CNTFAx15 decreased 24-h food intake and body weight in rats on HF or LF diet and increased the phosphorylation of hypothalamic S6K1 and S6 in a comparable way on both diets. Importantly, mice lacking the expression of S6K1 (S6K1-/-) did not respond to the anorectic action of either leptin or CNTFAx15, implying a crucial role for S6K1 in modulating the actions of these two cytokines. Finally, exposure to HF diet decreased mTORC1 signaling within the hypothalamus (Figure 5) and increased mTOW signaling in hippocamous (Figure 6). Overall, these findings strongly point to the possibility that reduced hypothalamic mTORC1 signaling contributes to the development of hyperphagia, weight gain and leptin resistance during diet-induced obesity.

Project 2 Year 2 Tasks:

Task 2: Measure cognitive and behavioral effects of chronic orexin-A activation (months 13-24)

- Assess the effects of chronic delivery of modafinil on memory, stress, motoric ability and perception (months 17-24)
- Identify and assess the effects of chronic delivery of secondary pharmacological targets from Project 1 (months 17-36).

Experiment Series 1.2 Methods

In the first series of experiments, rats received acute administration of modafinil (0.1 and 1.0 nmole/1 μl) or vehicle 1-hr prior to training in standard memory tasks for rodents: radial arm maze task and the passive avoidance task (details described below). In the second series of studies, we investigated the effects of chronic modafinil on response to stressful stimuli.

Passive avoidance test: Rats received daily ICV 0.1, 1.0 nmoles modafinil or vehicle for 2 weeks prior to training in the passive avoidance task. In this task, rats are presented with a moderate tone on one side of a conditioning chamber. When rats "escape" the presence of this tone by moving into the opposite chamber, the doorway is closed and rats receive a mild foot-shock. They are returned to the apparatus after 24 hours for testing. In both instances, we measure the latency to enter the opposite side of the chamber to avoid the moderate tone stimulus. An increase in latency indicates memory of the footshock.

Elevated plus maze (EPM): We have also administered daily ICV (0.1 nmoles and 1.0 nmoles) modafinil or vehicle for 2 weeks prior to acute testing in critical measures of stress and anxiety. The EPM test is a standard measures of stress and anxiety in rats and mice. Here, we assessed the effects of modafinil to increase time spent in the closed arm of the EPM (as an index of stress). An elevated plus maze constructed of 1/8" polypropylene plastic was used. Each of four arms (10 x 50 cm) is adjoined by a 10 x 10 cm intersection. The base of the maze is constructed such that the arms are elevated 50 cm above the ground. Animals were placed in the center of the apparatus facing an open arm and allowed to freely explore the apparatus for 5 min for behavioral observation. Tests were conducted at lights-out (the time of greatest activity for rodents) and both tests were 20 minutes in duration.

Intraventricular Cannulation. Animals were shaved and surgically prepped. A 2-cm midsaggital skin incision was made to expose the skull. Holes for anchoring screws and the cannula were drilled. A stainless steel (22 gauge, Plastics One) guide cannula extending into the third ventricle was permanently affixed to the skull by means of metal bone screws and quickly-drying dental acrylic. A removable 18 gauge obdurator sealed the guide cannula when not in use. All skull openings are sealed with dental acrylic. Gelfoam or bone wax followed by skin closure with suture. By manipulating the placement of the cannula, we can also put the cannula into specific brain regions for more local injection of substances.

Experiment Series 2.1 Methods

Working / Reference Memory Assessment: In this task, 4 of the 8 arms in an 8-arm radial arm maze are baited with food (see diagram), all arms are open for the animal to enter and remain open for the entire trial. All arms are identical, requiring the animal to utilize spatial cues external to the maze to identify and recall the location of the food. The animals were placed in the center of the maze and allowed to explore freely until all 4 food pellets have been consumed. Rats received chronic ICV infusions of the mTOR inhibitor, rapamycin or vehicle during training.

Experiment Series 1.2 Results

We observed little or no significant effects of modafinil (either dose) to increase anxiety-like behavior in rats. In the EPM test, experimental rats exhibited levels of anxiety-like behavior similar to those observed in vehicle treated rats (Figure 7). Specifically, chronic modafinil did not increase significantly the amount of time spent in the closed arm. Chronic modafinil did not impair performance in the passive avoidance test (Data not shown). The statistical significance of the data were analyzed by 1-way between-subjects ANOVA and Tukey's HSD post-hoc tests. Asterisks indicate statistically significant differences from vehicle treated rats.

Experiment Series 1.2 Results

Finally, we did observe that chronic inhibition of the mTOR pathway attenuated reference, but not working memory (Figures 8 & 9). The statistical significance of the data were analyzed by 1-way between-subjects ANOVA and Tukey's HSD post-hoc tests. Asterisks indicate statistically significant differences from vehicle treated rats.

Key Research Accomplishments:

- Chronic modafinil reduces body weight with a transient decrease in food intake.
- Chronic modafinil does not attenuate glucose tolerance or insulin sensitivity..
- Acute ICV modafinil does not increase stress or anxiety levels in rats.
- Acute ICV modafinil may impair hippocamal- but not amygdale-dependent memory.
- Nutrients acutely activate the mTOR pathways in the CNS.

Reportable Outcomes:

1. Portions of the Year 1 data were presented at the 2007 annual meeting of the Society for Neuroscience in San Deigo, CA.

2. Portions of the Year 2 data were presented at the 2008 annual European Winter Conference for Brain Research (Les Arc, France).

3. No patents or cells lines have been developed.

4. An animal model (mouse) of chronic variable stress based on the hypotheses generated here is currently under development in collaboration with Dr. James Herman (University of Cincinnati).

5. The data collected in Years 1 and 2 are contained in a manuscript that is being prepared for submission.

Conclusions:

While, the studies of Year 2 were by design more descriptive than mechanistic, we were able to draw several important conclusions that will guide the execution of experiments proposed for the subsequent years' funding periods. First, we conclude that chronic intraperitoneal administration of modafinil does not increase body adiposity in rats or impair glucose tolerance. Second, we conclude that *chronic* administration of modafinil directly into the central nervous systems *does not* in itself increase stress or anxiety-like behaviors. These data were critical for the correct interpretation of data to be colleted in experiments during the subsequent funding periods. We are now focused on a potential mechanistic aspect of these studies by assessing the effects mTOR activation. These studies are ongoing..

References:

1. Woods, S.C., Seeley, R.J., Rushing, P.A., D'Alessio, D.A., and Tso, P. 2003. A controlled high-fat diet induces an obese syndrome in rats. *Journal of Nutrition* 133:1081-1087.

Appendices:

n/a

Supporting Data:

See next pages.

Figure 1.

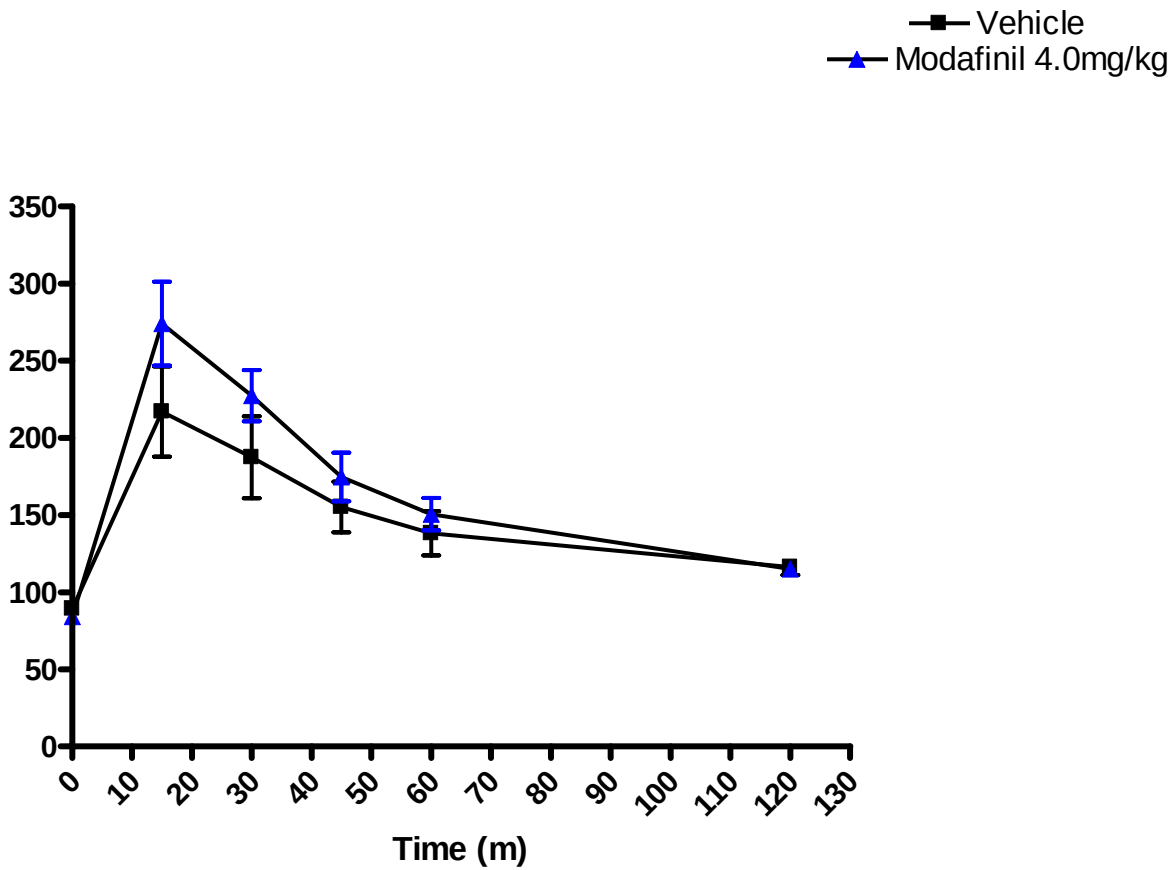

Vehicle
Modafinil 4.0mg/kg
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Time (m)

Figure 2.

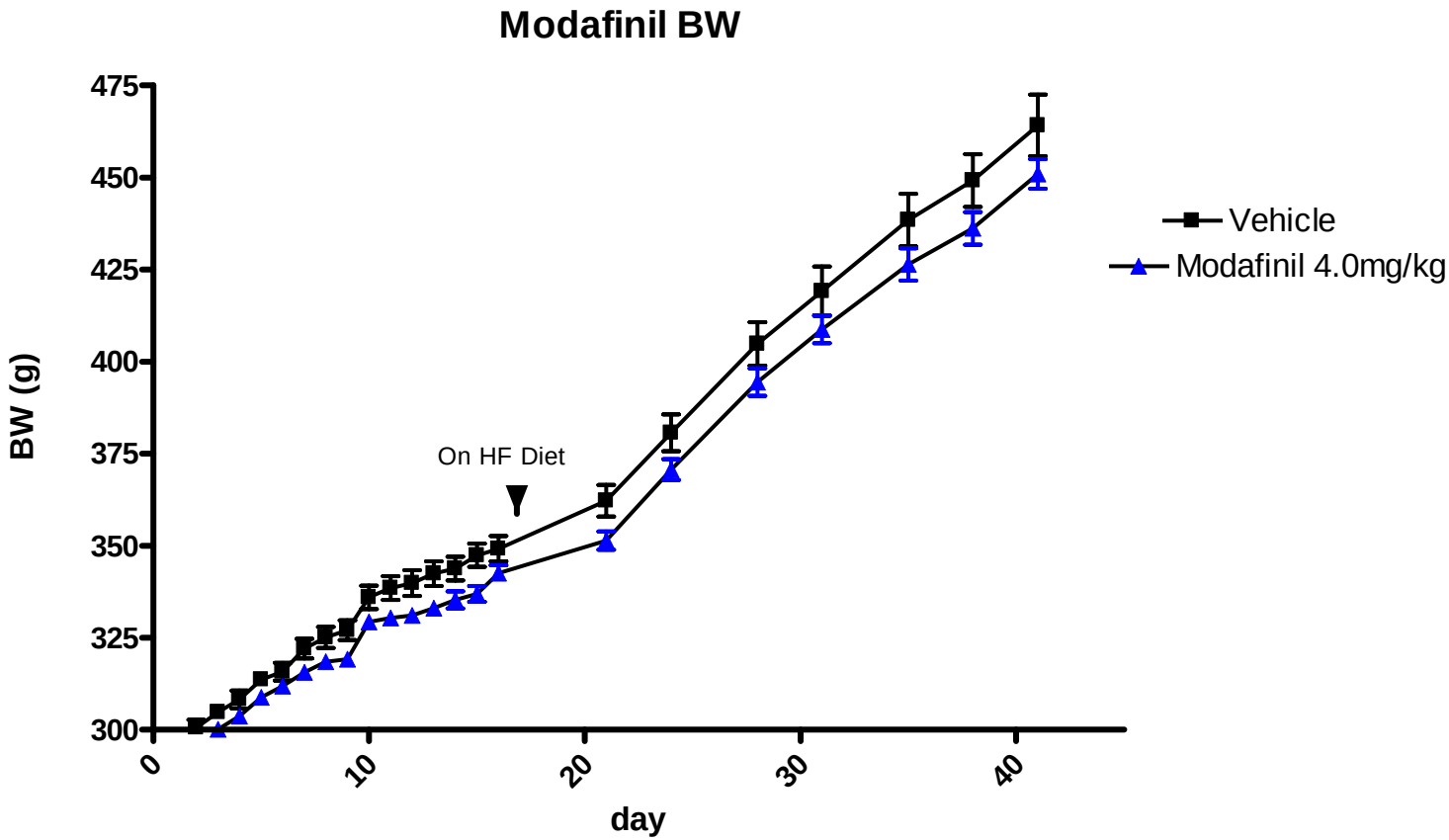

Modafinil BW
BW (g)
475
450
425
400
375
350
325
300
0
10
20
30
40
day
On HF Diet
Vehicle
Modafinil 4.0mg/kg

Figure 3.

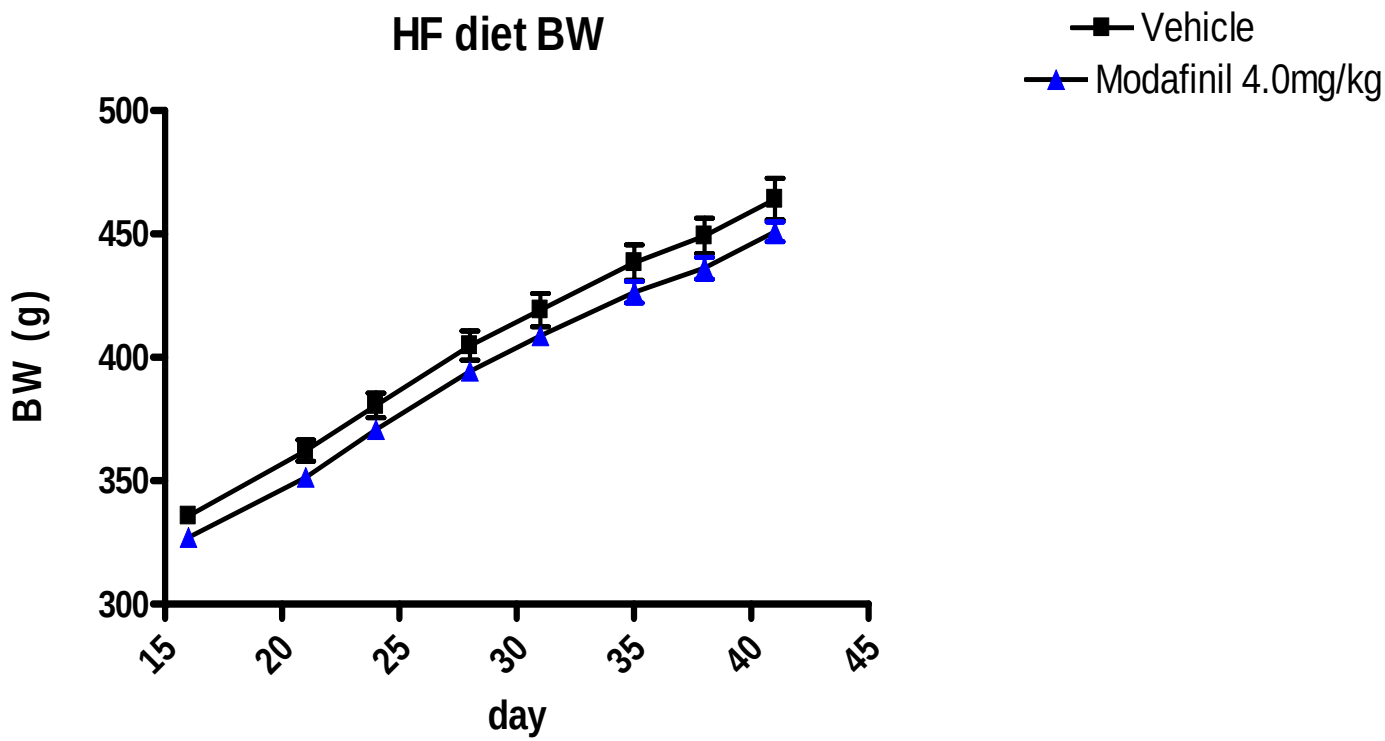

HF diet BW
Vehicle
Modafinil 4.0mg/kg
BW (g)
500
450
400
350
300
15
20
25
30
35
40
45
day

Figure 4.

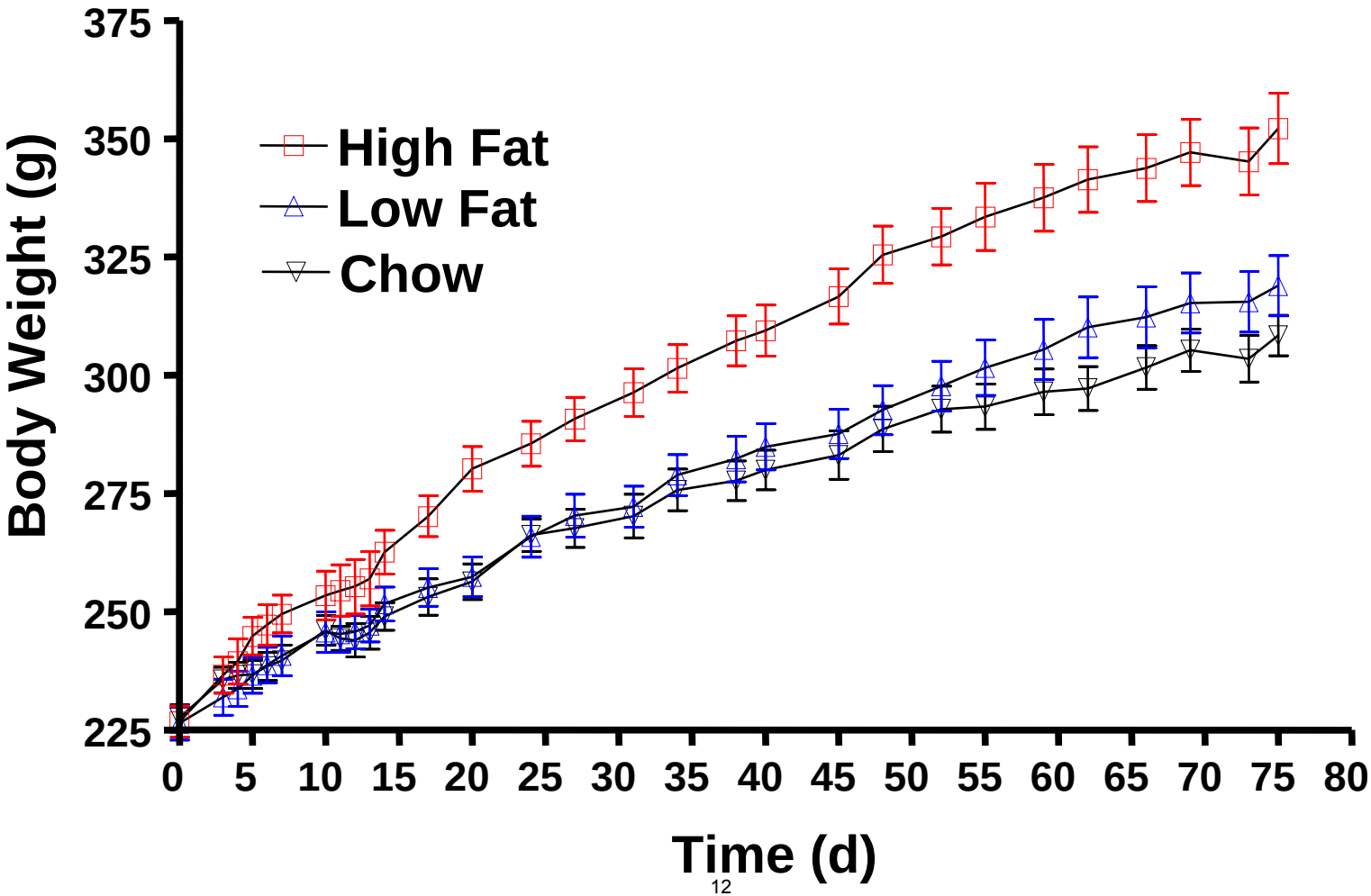
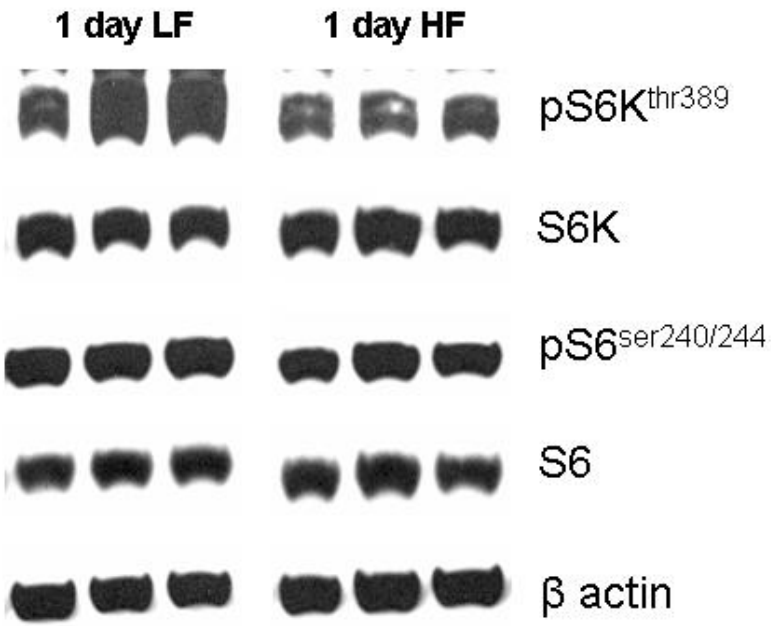
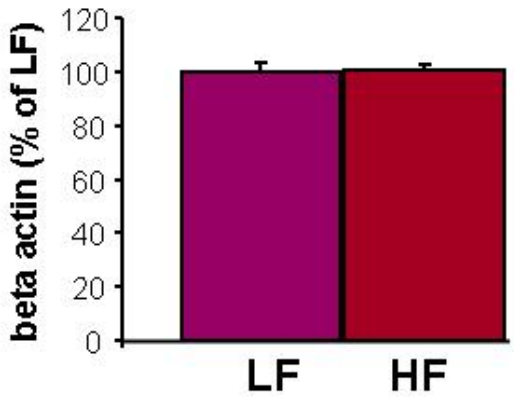
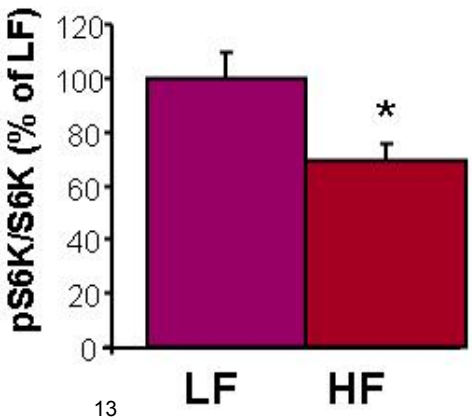

Body Weight (g)
375
350
325
300
275
250
225
High Fat
Low Fat
Chow
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
Time (d)

Figure 5.


1 day LF
1 day HF
pS6K thr389
S6K
pS6 ser240/244
S6
β actin


beta actin (% of LF)
120
100
80
60
40
20
0
LF
HF


pS6K/S6K (% of LF)
120
100
80
60
40
20
0
*
LF
HF

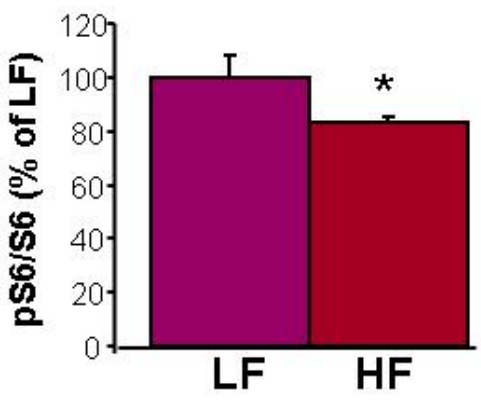

pS6/S6 (% of LF)
120
100
80
60
40
20
0
*
LF
HF

Figure 6.

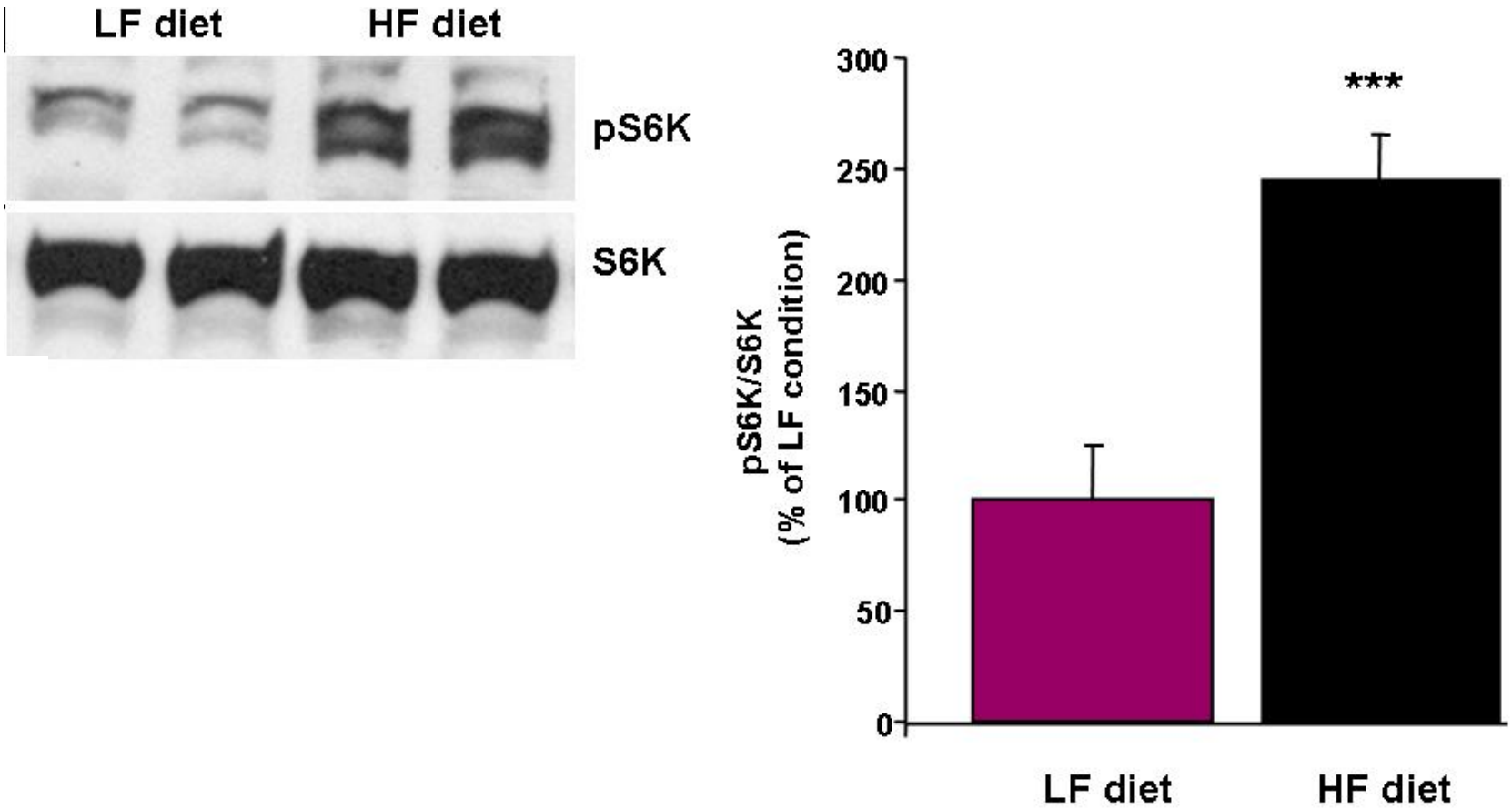

LF diet
HF diet
pS6K
S6K
pS6K/S6K
(% of LF condition)
300
250
200
150
100
50
0
***
LF diet
HF diet

Figure 7.

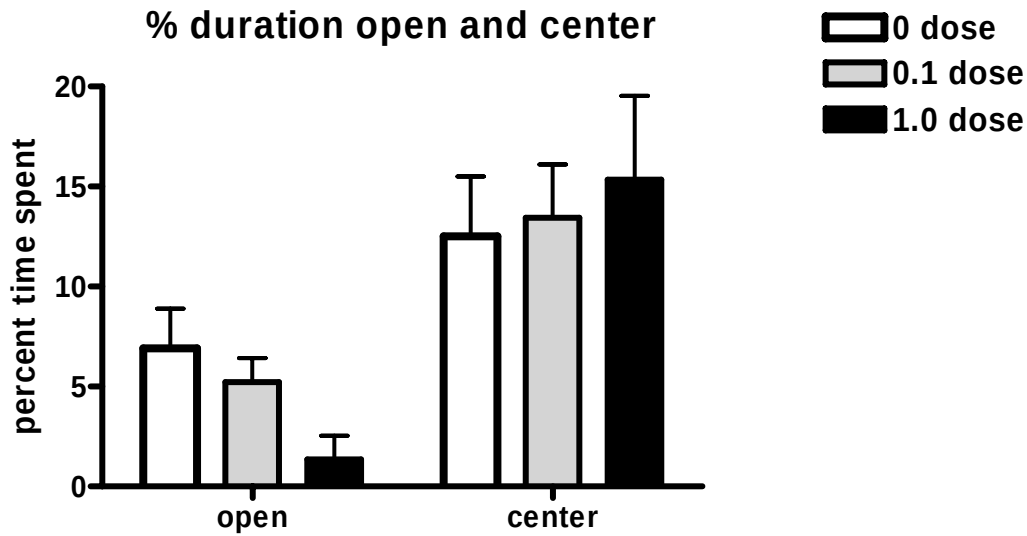
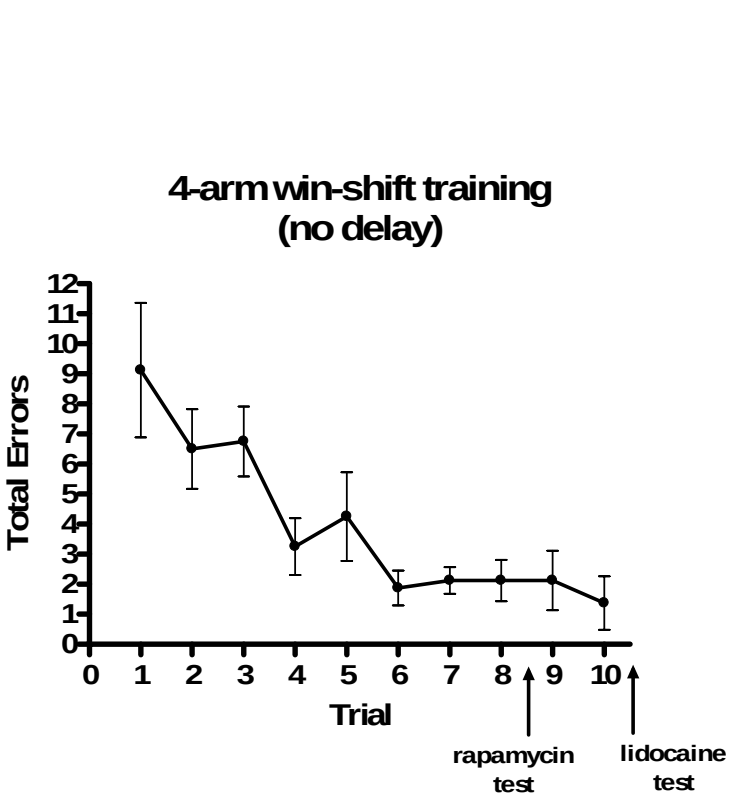
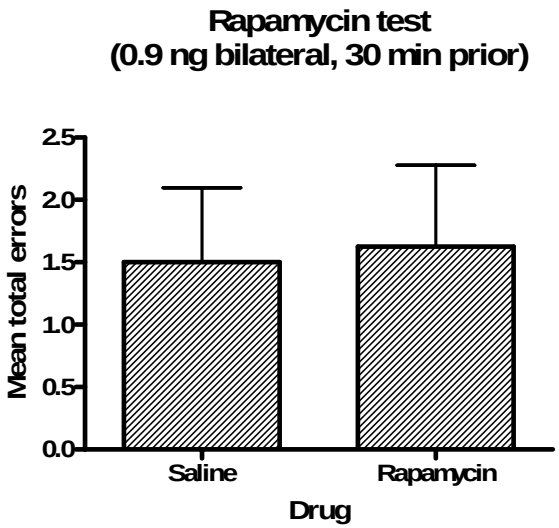

% duration open and center
percent time spent
20
15
10
5
0
open
center
0 dose
0.1 dose
1.0 dose

Figure 8.


4-arm win-shift training
(no delay)
Total Errors
0
1
2
3
4
5
6
7
8
9
10
11
12
0 1 2 3 4 5 6 7 8 9 10
Trial
rapamycin test
lidocaine test


Rapamycin test
(0.9 ng bilateral, 30 min prior)
Mean total errors
0.0
0.5
1.0
1.5
2.0
2.5
Saline
Rapamycin
Drug

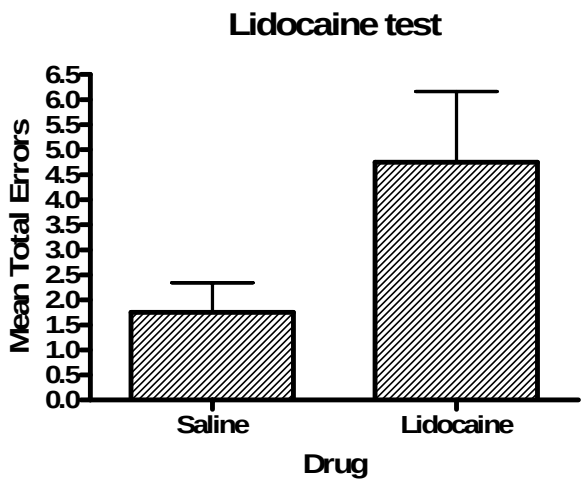
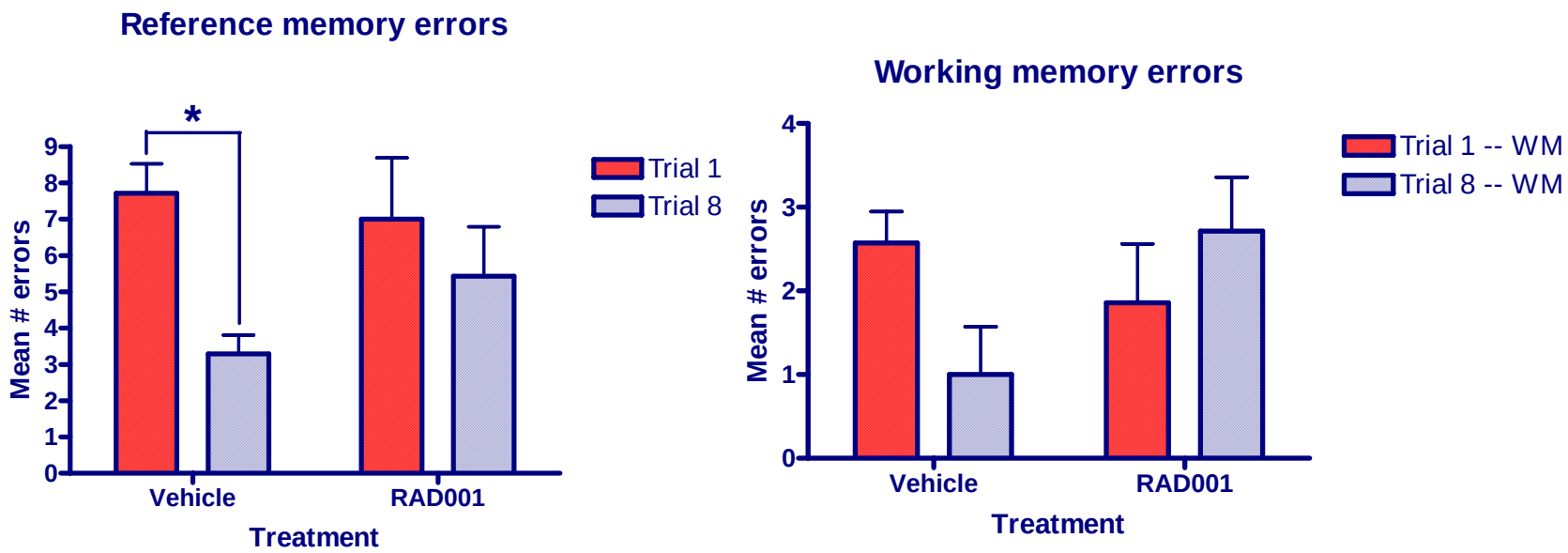

Lidocaine test
Mean Total Errors
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Saline
Lidocaine
Drug

Figure 9.


Reference memory errors
*
Mean # errors
0
1
2
3
4
5
6
7
8
9
Vehicle
RAD001
Treatment
Trial 1
Trial 8
Working memory errors
Mean # errors
0
1
2
3
4
Vehicle
RAD001
Treatment
Trial 1 -- WM
Trial 8 -- WM